96-well Serum/Plasma Fatty Acid and Glycerol Kit for the Detection of Both Non-Esterified Fatty Acids and Free Glycerol

Cat# GFA-1

INSTRUCTION MANUAL ZBM0033.05

STORAGE CONDITIONS

- **Reagents & Buffers:** 4°C **Use reconstituted Glycerol Reagent A within 7 days.**
- **Blank assay plates (96-well):** Room Temperature

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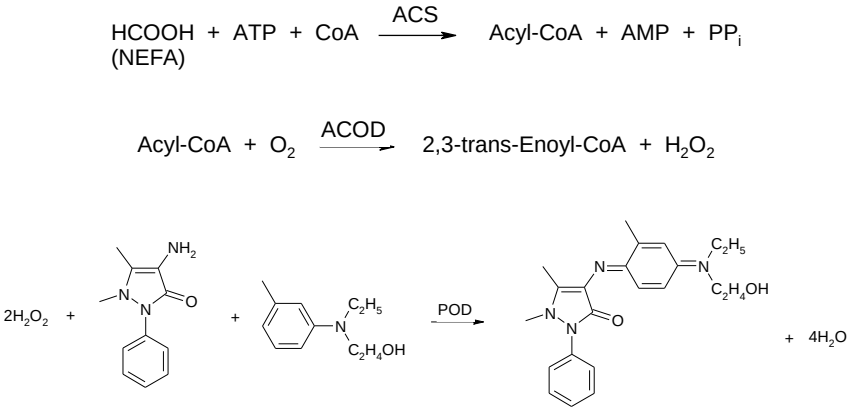
INTRODUCTION

This kit is designed to accurately determine the amount of free fatty acid and glycerol present in blood serum or plasma of humans, mice, rats, and other animals in a 96-well format for increased throughput analysis. Blood can be collected in plain evacuated tubes or in the presence of common anti-coagulants: sodium citrate, ammonium oxalate and EDTA. **NOTE: Heparin or Heparinized tubes should not be used because this will generate inaccurate readings.** Serum should be separated from clotted blood by centrifugation as soon as possible and may be stored frozen (-20°C) prior to analysis.

PRINCIPLES OF THE ASSAYS

Detection of Non-Esterified Fatty Acids (Free Fatty Acids; FFA)

Assessment of serum fatty acids is through a coupled reaction to measure non-Esterified fatty acids (NEFA). The initial step, carried out by acyl-CoA synthetase (ACS), produces fatty acyl-CoA thiol esters from the NEFA, ATP, Mg, and CoA in the reaction. The acyl-CoA derivatives react with oxygen in the presence of acyl-CoA oxidase (ACOD) to produce hydrogen peroxide. Hydrogen peroxide in the presence of peroxidase (POD) allows the oxidative condensation of 3-methyl-N-ethyl-N-(β-hydroxyethyl)-aniline with 4-aminoantipyrine which forms a purple product that absorbs light at 550nm. This allows the concentration of NEFA to be determined from the optical density measured at 540 - 550nm.



Detection of Free Glycerol

Glycerol present in sample is phosphorylated by adenosine triphosphate (ATP) forming glycerol-1-phosphate (G-1-P) and adenosine-5'-diphosphate (ADP) in the reaction catalyzed by glycerol kinase. G-1-P is then oxidized by glycerol phosphate oxidase to dihydroxyacetone phosphate (DAP) and hydrogen peroxide (H_2O_2). A quinoneimine dye is produced by the peroxidase catalyzed coupling of 4-aminoantipyrine (4-AAP) and sodium N-ethytl-N-(3-sulfopropyl)m-anisidine (ESPA) with H_2O_2, which shows an absorbance maximum at 540nm. The increase in absorbance at 540nm is directly proportional to glycerol concentration of the sample.

GLYCEROL + ATP ⟶ G-1-P + ADP

G-1-P + O_2 ⟶ DAP + H_2O_2

H_2O_2 +4-AAP + ESPA ⟶ Quinoneimine dye + H_2O

ITEMS INCLUDED IN THE KIT

ITEM	DESCRIPTION	Cap Color	UNIT	QTY	STORAGE
Assay Plate, blank	96-well assay plate, blank	---	PLATE	3	-----
Dilution Buffer	50 ml	---	BOTTLE	1	4°C
FFA Standard	1mM Stock. See page 4 for standard curve preparation	AMBER	100 µl / VIAL	1	4°C
FFA Diluent A		YELLOW	10.5 ML	1	4°C
FFA Diluent B		PINK	5.5 ML	1	4°C
FFA Reagent A	Reconstitute using 10.5 ml FFA Diluent A. Discard remainder after 10days	YELLOW	BOTTLE	1	4°C
FFA Reagent B	Reconstitute using 5.5 ml FFA Diluent B. Discard remainder after 10 days	PINK	BOTTLE	1	4°C
Glycerol Reagent A (cat# RGTA-10)	Reconstitute with 11.0 ml deionized water prior to use. Use within 7 days.	---	BOTTLE	1	4°C
Tray	For multi-channel pipetters, clear polyvinyl	CLEAR	EACH	2	-----
Glycerol standard (cat# LIP-GLYSTAN)	Glycerol @ 1mM [Dilute with 400 µl Dilution Buffer to make the 200 µM glycerol standard; see page 5 for recommended dilution scheme]	ORANGE	100 µl / VIAL	1	-20°C

Other equipment/reagents required but not provided with the kit:

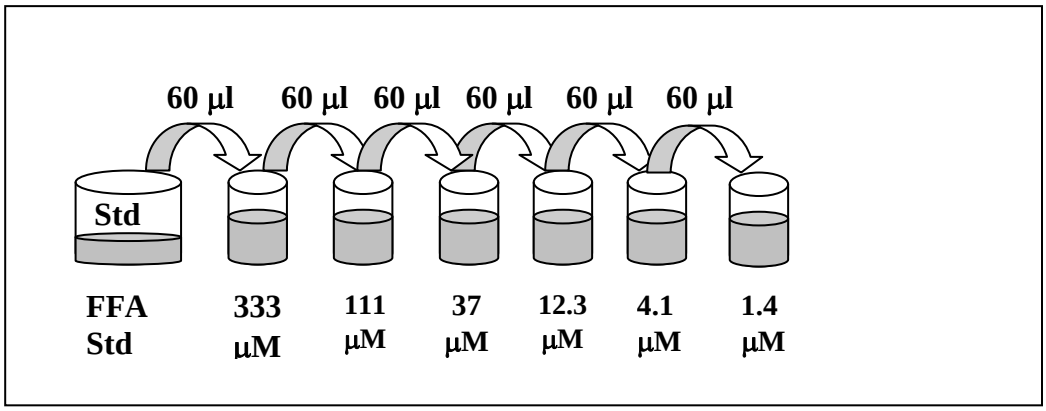
- Multi-channel Pipet , single channel pipet and pipet tips
- Plate reader with a filter of 540 nm
- Incubator at 37°C
- Large gauge needle
- Tubes for diluting standards

ASSAY PROCEDURE

A. DETECTION OF NON-ESTERIFIED FATTY ACIDS

1. Prepare the NEFA standard curve using the FFA STANDARD SOLUTION as follows:

   Briefly spin down the contents of the free fatty acid standard tube. Standards are: 0, 1.4, 4.1, 12.3, 37, 111, and 333 μM fatty acid. Prepare as follows:
   The kit standard solution is the 1.0 mM standard. Pipette 120 μl of Dilution Buffer into 6 tubes (not provided). Pipette 60 μl of the FFA Standard Stock into a tube labeled 333 μM. Prepare a dilution series as depicted below. Mix each new dilution thoroughly before proceeding to the next. The Dilution Buffer alone serves as the zero standard.



Note: The above dilution series generates enough volume to perform the standard curve in duplicate. If you wish to perform the standard curve in duplicate, please note that seven fewer data points can be assayed with this kit.

2. Also at this time prepare the FFA Reagent A by adding 10.5 ml FFA Diluent A per bottle and gently inverting. DO NOT VORTEX! Store any remaining solution at 2-8°C; it is stable for 10 days after reconstitution refrigerated (2-8°C).
3. Add 5 μl (or 1 - 10 μl) of serum or plasma to a well of Plate A. Add dilution buffer to each well to total 50 μl including serum or plasma sample. **THIS RESULTS IN A 10x DILUTION OF YOUR SAMPLE (5 μl in 50 μl).** Add 50 μl of each standard to empty wells (use PLATE B if necessary).
4. Add the reconstituted FFA Reagent A to one of the disposable trays provided in the kit. Add 100 μl of FFA Reagent A to each well. Gently shake the plate to ensure mixing. Place in a 37 °C incubator for 10 minutes.
5. Prepare the FFA Reagent B by adding 5.5 ml FFA Diluent B per bottle and gently inverting. DO NOT VORTEX! Store any remaining solution at 2-8°C; it is stable for 10 days after reconstitution refrigerated (2-8°C).
6. Add the reconstituted FFA Reagent B to the other disposable tray provided in the kit. Add 50 μl of FFA Reagent B to each well. Gently shake the plate to ensure mixing. Place in a 37 °C incubator for 10 minutes.

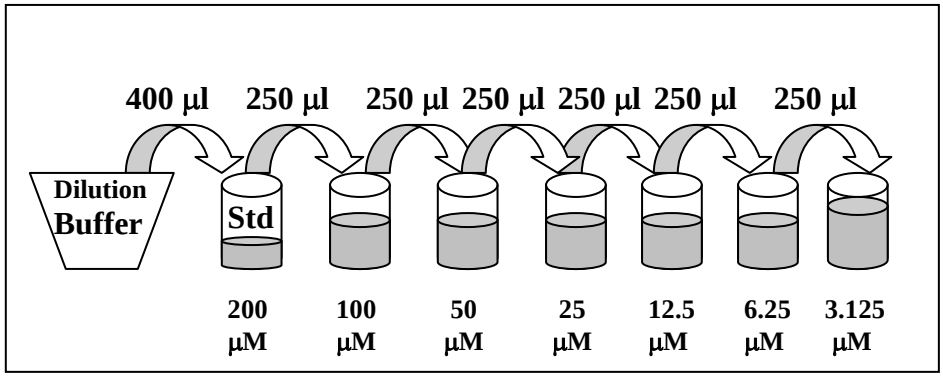
7. Allow the plate to equilibrate to room temperature for 5 minutes. During this time, ensure that there are no bubbles in the solution mixture. Use a large gauge needle or clean pipet tip to pop any bubbles as this will result in inaccurate absorbance readings.

8. The optical density of each well is then measured at 540 nm.

B. DETECTION OF FREE GLYCEROL

1. Prepare the glycerol standards as follows:

   Briefly spin down the contents of the glycerol standard tube before reconstitution. Pipette 400 µl of Dilution Buffer into the 1 mM glycerol standard tube provided and mix well by vortexing. This produces a diluted stock glycerol standard of 200 µM. Pipette 250 µl of dilution buffer into 6 tubes (not provided). Using the newly diluted stock glycerol solution, prepare a dilution series as depicted below. Mix each new dilution thoroughly before proceeding to the next. The 200 µM stock dilution serves as the highest standard, and the dilution buffer serves as the zero standard.



Note: The above dilution series generates enough volume to perform the standard curve in duplicate. If you wish to perform the standard curve in duplicate, please note that eight fewer data points can be assayed with this kit.

2. Also at this time prepare the Glycerol Reagent A by adding 11.0 ml room temperature deionized water per bottle and gently invert. DO NOT VORTEX! Use a pipet to ensure that the powder is completely dissolved. Store at room temperature. If using a Reagent A solution previously prepared and stored at 2-8°C, also bring to room temperature. Make sure there is enough Reagent A from one solution to treat all the points in the assay. It may be necessary to combine solutions. Store in a light protected bottle. Reconstituted Glycerol Reagent A is stable for 7 days refrigerated (2-8°C); store any remaining solution refrigerated (2-8°C).

3. Add 20 µl (or 10 - 25 µl) of serum or plasma to a well of a blank plate for assessment of free glycerol. Add 30µl of dilution buffer to each well to total 50 µl including serum or plasma sample. **THIS RESULTS IN A 2.5x DILUTION OF YOUR SAMPLE (20 µl in 50 µl).** Add 50 µl of each standard to empty wells (use another plate, if necessary).

4. Add the reconstituted Glycerol Reagent A solution to one of the disposable trays provided in the kit. Add 50 µl of Reagent A to each well of the glycerol plate. Gently, pipet up and down once to mix. Pop the

bubbles using a large gauge needle or a clean pipet tip. The plate is then incubated at 25°C (room temperature) for 15 minutes.

5. The optical density of each well is then measured at 540 nm.

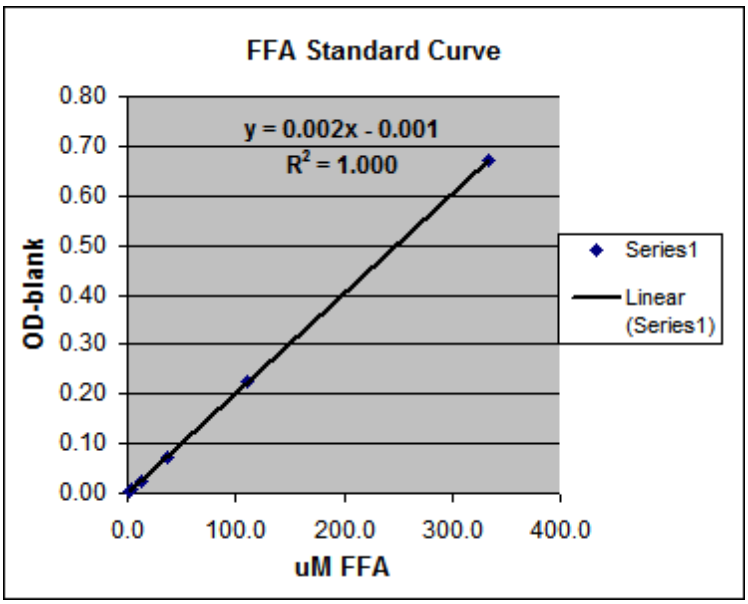
FATTY ACID STANDARD CURVE _____

Generate standard curve: see example below

[DO NOT use this standard curve to generate your data. This is an example.]

Subtract the OD value of the 0μM standard from all OD values including the standard curve. . Note: 1mM standard is commonly omitted from analysis due to lack of linearity between 333 μM and 1mM. Optionally, a 4-parameter fit may be used to calculate standard curve.

uM FFA	OD	OD	OD-blank	OD-blank	Avg OD-blank
0	0.05	0.048			0.049
1.4	0.051	0.053	0.002	0.004	0.003
4.1	0.056	0.058	0.007	0.009	0.008
12.3	0.070	0.075	0.021	0.026	0.024
37	0.119	0.122	0.070	0.073	0.072
111	0.274	0.277	0.225	0.228	0.227
333	0.689	0.750	0.640	0.701	0.671



Slope	0.002
Intercept	-0.001
R^2	1.000

y = observed O.D. minus the blank

x = concentration of FFA in μM

To calculate x for each y, (i.e. to change the observed O.D. into FFA concentration) use the following equation:

y=(slope) times (x) plus intercept

y=mx+b so x=(y-b)/m

x=(y – (-0.001))/0.002 where 0.002= slope of the line and -0.001= y intercept. Be careful to enter the proper sign for the y intercept value as it may be a negative number.

Data are expressed as μM free fatty acids released.

OPTION: express data as Fold induction over appropriate vehicle

$$\text{Fold induction} = \frac{\mu\text{M free fatty acids SAMPLE}}{\mu\text{M free fatty acids VEHICLE}}$$

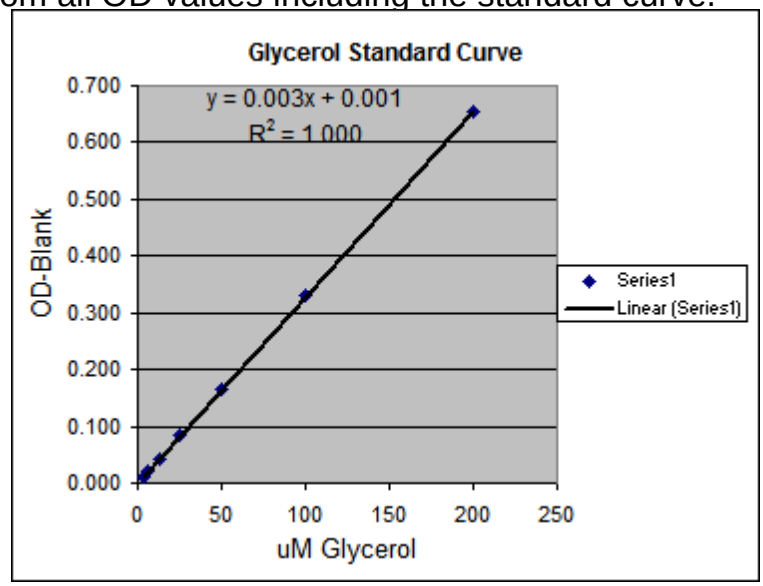
The R^2 value should be equal or greater then 0.98 for the standard curve to be valid. Any R^2 values below 0.98 must have the standard curve run again.

GLYCEROL STANDARD CURVE _____

Generate standard curve: see example below
[DO NOT use this standard curve to generate your data. This is an example.]

Subtract the OD value of the 0μM standard from all OD values including the standard curve.

uM glycerol	OD	OD	OD-blank	OD-blank	Avg OD-blank
0	0.044	0.041			0.043
3.125	0.054	0.053	0.012	0.011	0.011
6.25	0.062	0.063	0.020	0.021	0.020
12.5	0.083	0.084	0.041	0.042	0.041
25	0.126	0.125	0.084	0.083	0.083
50	0.205	0.208	0.163	0.166	0.164
100	0.372	0.374	0.330	0.332	0.331
200	0.698	0.697	0.656	0.655	0.655



Slope	0.003
Intercept	0.001
R^2	1.000

y = observed O.D. minus the blank
x = concentration of glycerol in μM
To calculate x for each y, (i.e. to change the observed O.D. into glycerol concentration) use the following equation:

y=(slope) times (x) plus intercept

y=mx+b so x=(y-b)/m

x=(y – (0.001))/0.003 where 0.003= slope of the line and 0.001= y intercept. Be careful to enter the proper sign for the y intercept value as it may be a negative number.

Any OD values greater than the highest standard (200 μM) should be suspect. The compound should be re-assayed using a lower dose of the compound at treatment OR a dilute solution of the condition medium at the time of the assay.

The R^2 value should be equal or greater than 0.98 for the standard curve to be valid. Any R^2 values below 0.98, must have the standard curve run again.

Data are expressed as μM glycerol.

REMEMBER TO ACCOUNT FOR THE DILUTION FACTOR IN STEP 3.

FREQUENTLY ASKED QUESTIONS: _____

1. **Can I buy the reagents separately?** The Glycerol Standard, cat# LIP-GLYSTAN, Free fatty Acid Standard, cat# FFA-STAN and Glycerol Reagent A, cat# RGTA-10 are sold separately. LIP-2/3 Assay Buffer, Free Fatty Acid Reagents and Diluents A and B are not sold separately.

2. **I need to know the concentration of the BSA in the Assay Buffer?** ZenBio, Inc does not provide the concentrations of the components of our media and buffers. If knowledge of the BSA concentration is critical to your experiment, you may order Assay Buffer WITHOUT BSA for no additional charge. Please note it on your order.

3. **What is the Free Fatty Acid standard?** Free Fatty Acid standard (cat# FFA-STAN) is oleic acid in an aqueous buffer.

4. **I have more samples plus standards to run than can fit on 1 96 well plate. Can I compare data obtained from multiple plates?** The lipolysis kit is designed for the assay of a single plate. You may purchase 2 kits of the same lot number. You may then use one plate that includes the blank, vehicle(s), and positive and negative controls. The second plate may then be used for the remainder of your samples assayed. In order to obtain comparable data, both plates must be assayed on the same day using kits and cells from the same lot number. An additional blank assay plate is provided for the assay of glycerol standards.

APPENDIX A: PLATE LAYOUT _____

H	G	F	E	D	C	B	A	
								1
								2
								3
								4
								5
								6
								7
								8
								9
								10
								11
								12

APPENDIX B: PROCEDURE FLOWCHART

NEFA Detection

Add 5 µl/well test sample and 45 µl/well dilution buffer to one of the blank assay plates provided.
Add 50 µl/well diluted standard curve to empty wells.

↓

Reconstitute FFA Reagent A using Diluent A.
Add 100µl/well. Incubate 10 minutes @ 37°C.

↓

Reconstitute FFA Reagent B using Diluent B.
Add 50µl/well. Incubate 10 minutes @ 37°C.

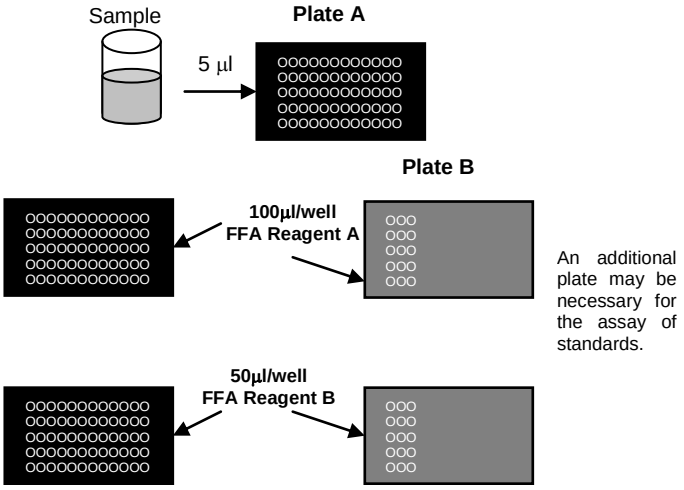
↓

Place at room temp. for 5 minutes. Pop any bubbles in each well using a clean pipet tip or large gauge needle.

↓

Measure the optical density of each well at 540 nm using a spectrophotometer plate reader.

Reminder: Sample was diluted in Step 3



An additional plate may be necessary for the assay of standards.

Glycerol Detection

Add 20 µl/well test sample and 30 µl/well dilution buffer to one of the blank assay plates provided.
Add 50 µl/well diluted standard curve to empty wells.

↓

Reconstitute Glycerol Reagent A.
Add 50µl/well.

↓

Incubate 15 minutes @ room temperature.

↓

Measure the optical density of each well at 540 nm using a spectrophotometer plate reader.

Reminder: Sample was diluted in Step 3